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The N-Terminal Half of EBNA2, except for Seven Prolines, Is Not Essential for Primary B-Lymphocyte Growth Transformation

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Previous molecular genetic analyses of Epstein-Barr virus nuclear protein 2 (EBNA2) identified a negative effect of deletion of codons 19 to 33 on transformation and gene transactivation, while deletion of codons 19 to 110 was a null mutation for transformation and gene transactivation. We here report the surprising finding that codons 2 to 88, which encode the highly conserved unique N terminus (amino acids 1 to 58) and most of the polyproline repeat (amino acids 59 to 95), can be deleted with only minimal effects on transformation. Codons 97 to 122 can also be deleted with only minimal effects on transformation. However, deletion of 35 of the 37 prolines (amino acids 59 to 93) or deletion of codons 2 to 95 results in a null transforming phenotype. Although EBNA2 from which codons 59 to 93 were deleted was a null mutation for transformation, it was similar to some transforming mutants of EBNA2 in abundance, in interaction with RBPJK, and in transactivation of the LMP1 promoter in transient transfection assays. These data indicate that between three and seven prolines are critical for EBNA2 structure or for intermolecular interaction. Aside from these seven prolines, codons encoding the rest of the N-terminal half (amino acids 2 to 230) of EBNA2 are nonessential for primary B-lymphocyte growth transformation.

Epstein-Barr virus (EBV) nuclear protein 2 (EBNA2) is one of the first two proteins expressed from the EBV genome in B lymphocytes (1, 17) and is essential for the transformation of primary B lymphocytes into long-term lymphoblastoid cell lines (LCLs) (4, 9). At least part of EBNA2's essential role in lymphocyte transformation is as a transactivator of cellular and viral gene expression (5, 22, 23, 26). EBNA2 lacks sequence-specific DNA-binding activity and recognizes specific promoters through cellular proteins. RBPJK and PU.1 are cellular sequence-specific DNA-binding proteins that are important in conferring EBNA2 responsiveness (8, 10, 12, 24). Once bound by cellular proteins that are associated with specific sites in the viral or cellular genome, EBNA2 can recruit basal transcription factors to nearby promoters through its acidic activating domain. The EBNA2 acidic activating domain can interact with TAF40, TFIIB, or TFIIH (18, 20). In transformed B lymphocytes, a substantial fraction of the EBNA2 acidic domain is associated with a p100 coactivator that has affinity for TFIIE (19).

The EBNA2 domains important for cell growth transformation have been partially defined by linker insertion or deletional analyses of the EBNA2 open reading frame (ORF) (3, 21, 24). DNA with a mutated EBNA2 ORF has been compared with wild-type (WT) DNA for the ability to marker rescue transformation after transfection into cells infected with the P3HR-1 EBV strain. The EBNA2 ORF (and two adjacent exons of EBNA LP) is deleted from P3HR-1 EBV and is therefore unable to transform cells. When P3HR-1-infected cells are transfected with a WT EBV DNA fragment that spans the site of the EBNA2 deletion, homologous recombination between the transfected DNA and the P3HR-1 EBV genome results in a WT EBV genome which can transform primary B lymphocytes into LCLs. An initial analysis with 11 linker insertions and 15 deletions revealed four parts of EBNA2 that appeared to be essential for LCL outgrowth (3). Regions 1, 2, 3, and 4 were defined by deletion of codons 19 to 110, by a linker insertion after codon 320, by deletion of codons 333 to 425, and by deletion of codons 426 to 462, respectively. Deletion of codons 112 to 141 or 143 to 230 was compatible with transformation, albeit with negative effects on transformation efficiency, while deletion of codons 462 to 482 had a slight positive effect. Codons 2 to 18 were not evaluated. These data were compatible with the hypothesis that at least four EBNA2 domains are important for LCL outgrowth and for transactivation of virus or cell gene expression.

While region 4 is the core acidic transactivating domain (3, 18–20) and region 2 is the site that interacts with RBPJK (24), each subdomain of region 3 proved to be nonessential for transformation (21). The RG repeat subdomain of region 3 conferred an ability to interact with histones or nucleic acids, modulated the activity of the acidic transactivating domain, and contributed to transformation efficiency. All other parts of region 3 could be deleted with almost no effect on transformation (21). Thus, the apparently essential requirement for region 3 is due to the importance of the RG domain and the need for a spacer between the acidic domain and the RBPJK interactive domain. The surprising results of this analysis of region 3 led us to reexamine the apparently essential requirement for region 1 (codons 19 to 110).

MATERIALS AND METHODS

Cell culture. BJAB (14) is an EBV-negative lymphoma cell line. The P3HR-1 clone 16 cell line harbors a defective EBV strain (15). Both cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Cosmids and plasmids. The *Hin*dIII-*Cpo*I fragment of the W91 *Eco*RI A cosmid (corresponding to residues 48039 to 52589 of the B95-8 sequence) was subcloned into a plasmid vector, and the sequences of interest were deleted by

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PCR mutagenesis (11). The inside primers used to generate *d*2-88, *d*97-122, *d*2-95, and *d*59-93, are CTTTATCTGCCGCCAGCATGCCCCCAGGACCC CCACCACC and GGTGGTGGGGGTGGTGGGGGCATGCTGGCGGCAG ATAAAG, GTCCAGGCATCCCTCAGCTGAGGTGGTGGG and TGGAC GAATAGCTTCTGCTATGCGAATGCTTTGG, GCTTTATCTGTCTAGAT CATGCAGCGCAGGGATGCCTGGAC and CCCTGCGCTGCATGATCTA GACAGATAAAGCAAAACACAAG, and GGGGAAAACACGGGGGTGC CACCTCAGCGCAGGGATGC and GCATCCCTGCGCTGAGGTGGCAC CCCCGTGTTTTCCCC, respectively. Mutation *d*2-289 was generated by introducing through PCR mutagenesis an *Mun*I site immediately downstream of the EBNA2 start codon. The *Mun*I site facilitated ligation to the *Mun*I site at amino acid 289. Deletion *d*111-289 was generated by blunt-end ligation of the *Bam*HI and *Mun*I sites within the EBNA2 ORF. The mutated *Hin*dIII-*Cpo*I fragment was cloned back into the *Eco*RI A cosmid, or the *Fnu*DII-*Aha*III fragment was cloned into the eukaryotic expression plasmid pSG5 (Stratagene). PCR products used for cloning are verified by nucleotide sequencing. The reporter construct p-234/+40 LMP1 TKCAT was previously described (22).

Transformation assaying. WT or mutant *Eco*RI A cosmid DNAs were digested with *Eco*RI to release the insert from the vector. P3HR-1 cells were electroporated with 10 μg of cosmid DNA and 40 μg of pSVnaeIZ plasmid DNA at 200 V and 960 μF by using a Bio-Rad Genepulser. The transfected cells were diluted into 15 ml of RPMI medium with 10% fetal bovine serum, and the mixtures were incubated at 37°C. After 5 days, culture supernatant containing virus was filtered through a 0.45-μm-pore-size filter and used to infect freshly prepared human peripheral blood B lymphocytes (3, 24). The infected cells were plated at 50,000 cells in 150 μl of medium per well. The cells were fed once every 2 weeks with fresh medium. LCLs were usually macroscopically visible at 4 to 6 weeks. Cultures were maintained for at least 10 weeks.

Virus passage. LCLs were induced to enter the lytic replication cycle by transfecting the cells with a BZLF1 expression plasmid and treating the transfected cells with 20 ng of phorbol myristate acetate per ml. Five days after induction, virus was harvested, filtered, and used to infect freshly isolated human peripheral blood lymphocytes as described above.

CAT assay. BJAB cells (15×10^6) were electroporated with 5 μg of pUC-β-gal, 40 μg of pSG5 or pSG5 with WT or mutant EBNA-2, and 5 μg of reporter plasmid p-234/+40 LMP1 TKCAT. Cells were harvested after 2 days, and extract was used in chloramphenicol acetyltransferase (CAT) assays. CAT data were normalized for β-galactosidase activity (22).

PCR. The virus supernatant used to infect human peripheral blood B lymphocytes was used in PCR to compare the relative amounts of virus present in the samples from WT and mutant LCLs. Viral suspensions were serially diluted, denatured by incubation for 30 min at 95°C, and used in amplification assays.

Immunoprecipitation. BJAB cells were transiently transfected with pSG5 expression vectors coding for WT or mutant EBNA2. The cells were harvested 20 h posttransfection and were lysed in 1% Nonidet P-40 buffer. The cell lysates were immunoprecipitated with the anti-EBNA2 monoclonal antibody PE2. The immunoprecipitates were electrophoresed through polyacrylamide gels, blotted onto nitrocellulose filters, and probed with PE2 or anti-RBPJK-specific polyclonal rabbit antiserum. Detection was by chemiluminescence with reagents from Amersham.

RESULTS

The unique N terminus and most of the polyproline domain of EBNA2 are not critical for primary B-lymphocyte transformation; some prolines are critical. The EBNA2 N terminus is composed of 58 amino acids (Fig. 1) which are highly conserved between the two human EBV types (3, 6, 7) and well-conserved in the baboon lymphocryptovirus (13). Included in the N terminus are two conserved tyrosines which could be substrates for phosphorylation (16). The N terminus is followed by a polyproline repeat domain which is characteristic of all EBV isolates but varies widely in length; a prototype type 1 EBV strain, W91, has 37 prolines (6). Previously, a deletion of EBNA2 codons 19 to 110 was found to have a null transforming phenotype in P3HR-1 marker rescue experiments, while deletions of codons 19 to 33 or 112 to 141 were associated with reduced transformation efficiency and slow outgrowth of the infected LCLs (3). The role of codons 2 to 19 had not previously been examined.

To further assess the role of the highly conserved unique amino terminus (amino acids 1 to 58) and most of the polyproline domain (amino acids 59 to 95), codons 2 to 88 were deleted from the EBNA2 ORF in two subclones (*d*2-88) of a WT EBV W91 DNA fragment that spanned the site of the P3HR-1 deletion. For comparison, codons 59 to 93 encoding

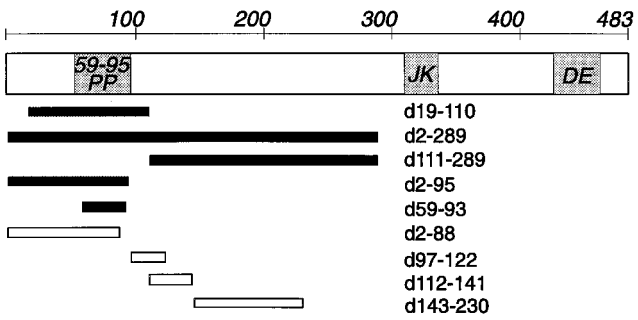

FIG. 1. A schematic map of EBNA2 indicating the deletions and their effects on EBV-mediated transformation of primary human B lymphocytes. The polyproline sequence (PP [residues 59 to 95]), RBPJK binding site (JK [residues 310 to 336]), and acidic transactivating domain (DE [residues 426 to 462]) are indicated. EBNA2 deletions that have been studied are indicated below the EBNA2 schematic map. Empty boxes indicate deletions that are compatible with primary B-lymphocyte transformation. Solid boxes indicate deletions that markedly affect the ability of EBV to transform B cells. *d*19-110, *d*112-141, and *d*143-230 were previously reported (3).

all but two of the prolines or codons 2 to 95 encoding the unique N terminus and the entire polyproline domain were deleted in other subclones of the WT EBV W91 DNA fragment (Fig. 1). Surprisingly, transfection of P3HR-1 cells with either of two clones of *d*2-88 DNA yielded LCLs. The efficiency of primary lymphocyte transformation marker rescue with virus stocks obtained following *d*2-88 transfection of P3HR-1-infected cells was approximately 10% of the efficiency of a WT stock derived in parallel (5, 7, or 8 transforming events with three independent *d*2-88 transfections versus 62 events with the WT control [Table 1]). These LCLs grew out as rapidly as LCLs transformed by the WT recombinants. Their growth properties remained similar to those of WT over several months in continuous culture.

In contrast to these results, two clones of *d*59-93 DNA and two clones of *d*2-95 DNA consistently failed to marker rescue transforming virus from P3HR-1 cells. Ten attempts at marker

TABLE 1. Efficiency of primary lymphocyte transformation marker rescue[a]

EBNA2 mutation	No. of LCLs with mutant virus	No. of LCLs with WT virus
*d*2-88	7, 5, 8	62
*d*59-93	0, 0, 0, 0	61
	0, 0	56
	0, 0	37
	0, 0	93
*d*2-95	0, 0	65, 63
	0, 0	36
	0, 0	48
*d*97-122	4, 8	60
	4, 5, 2, 3	96, 96
*d*2-289	0, 0	31
*d*111-289	0, 0, 0	56
	0, 0, 0	37
	0, 0, 0	31

[a] Each transfection transformation experiment included at least one WT cosmid and two or more independently derived mutant cosmids. Results from each experiment are indicated on a separate line.

TABLE 2. LCLs resulting from infection by EBNA2 mutants[a]

EBNA2 mutation	No. of LCLs with virus from individual mutant-infected LCLs (10^2)	No. of LCLs with virus from individual WT-infected LCLs (10^2)
*d*2-88	26, 0, 61, 0	93, 96, 0, 62
*d*97-122	80, 96, 80, 56	96, 92, 75, 39

[a] Each induction transformation experiment included four WT and four mutant LCLs. These first-generation LCLs were induced for the lytic replication cycle; the released virus was diluted 100-fold and used to infect human peripheral blood lymphocytes. The numbers of second-generation LCLs resulting from infection by each virus preparation are given.

rescue from P3HR-1-infected cells by transfection with *d*59-93 and six attempts with *d*2-95 yielded no LCLs; WT DNA control transfections in parallel consistently marker rescued at least 30 transforming EBV recombinants (Table 1). Since the nontransforming deletion *d*59-93 leaves two prolines and the transforming deletion *d*2-88 leaves seven prolines, these data indicate that at least three and perhaps as many as seven prolines are critical to EBNA2 functionality in primary B-lymphocyte growth transformation.

The 10% efficiency of marker rescue with *d*2-88 was somewhat surprising, considering the absence of an effect on the growth of *d*2-88-infected LCLs. The marker rescue experiments are done by transfection of P3HR-1 cells, and 10^5-fold as many nonrecombinant P3HR-1 EBV are produced, so that most of the recombinant infected cells are initially coinfected with P3HR-1 EBV. Since P3HR-1 EBV can inhibit transformation by WT EBV (15), we considered the possibility that the difference in transformation efficiency between *d*2-88 and WT recombinants appears to be larger than it is as a consequence of P3HR-1 coinfection. Therefore, we directly compared the transformation efficiencies of *d*2-88 and WT EBV recombinants. Virus replication was induced in four *d*2-88- and four WT recombinant-infected cell lines, and released virus was assayed by clonal transformation of primary lymphocytes (Table 2). Two of four *d*2-88 and three of four WT cell lines released virus. The best *d*2-88-infected cell line released 6,100 transforming recombinants, while the best WT-infected cell line released 9,600 recombinants. Both mutant and WT supernatants had similar levels of recombinant viral DNA as measured by end point dilution PCR (Fig. 2). The *d*2-88-infected LCLs grew indistinguishably from WT-infected LCLs. The similar transforming efficiencies for WT and *d*2-88-mutated

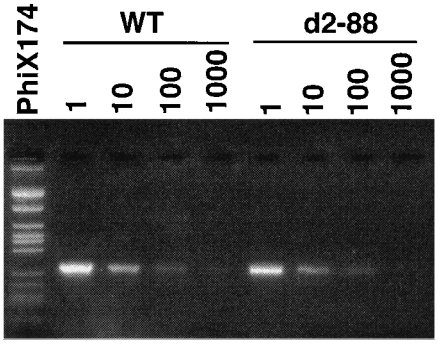

FIG. 2. EBNA2 *d*2-88 or WT EBV recombinant-transformed LCLs were induced to lytic infection, and filtered supernatants were assayed for viral DNA by serial dilution and PCR. Sequences corresponding to 49398 to 49670 of the B95-8 EBNA2 sequence (3) were amplified, since these were unaffected by the *d*2-88 deletion. Fold dilutions are indicated above each lane. PhiX174 DNA digested with *Rsa*I was used as molecular weight markers.

EBV recombinants and the similarity in growth of the mutant and WT transformed LCLs indicate that codons 2 to 88 are not critical for primary B-lymphocyte growth transformation in vitro or for virus replication in the resulting LCLs.

The highly conserved domain immediately C terminal to the polyproline domain is not critical for primary B-lymphocyte growth transformation in vitro. Since the highly conserved domain after the polyproline repeats (residues 97 to 110) had been evaluated only in the context of a larger deletion (*d*19-110 [3]) that included the polyproline domain which now appears to be critical for LCL outgrowth, we proceeded to evaluate the significance of the highly conserved domain by deleting codons 97 to 122 (Fig. 1) from the marker-rescuing DNA fragment. Two clones of *d*97-122 DNA marker rescued transforming virus from P3HR-1. Fewer *d*97-122 recombinant-transformed than WT-transformed B lymphocytes were obtained from marker rescue experiments (Table 1). However, the transformed B lymphocytes were indistinguishable in growth from WT recombinant-transformed lymphocytes. Further, when the resultant LCLs were induced to permissivity for virus replication, as much transforming EBV was produced as from LCLs infected with WT recombinants (Table 2). Thus, residues 97 to 122 are not critical for LCL outgrowth in vitro or for virus replication in the resultant LCLs.

Residues between codons 230 and 289 may be critical for primary B-lymphocyte transformation. The demonstration that codons 2 to 88 and 97 to 122 are not critical for primary B-lymphocyte growth transformation leaves only seven prolines which could account for the failure of *d*59-93, *d*2-95, and *d*19-110 to marker rescue. Further, EBNA2 from which codons 112 to 141 or 143 to 230 were deleted was still able to marker rescue transformation from P3HR-1 with reduced efficiency (3). Therefore, we considered the possibility that the RBPJK/PU.1 interactive domain, the acidic transactivating domain, and the spacer between them are the only really essential parts of EBNA2. The minimal RBPJK/PU.1 interactive domain begins at residue 310. Therefore, we investigated whether deletion of codons 2 to 289 or 111 to 289 would be compatible with marker rescue of transformation from P3HR-1-infected cells. Two different clones of *d*2-289 and three different clones of *d*111-289 failed to marker rescue transforming virus from P3HR-1 (Table 1). The failure of these two mutations to marker rescue transforming activity could be due to the importance of residues 231 to 289 or to a drastic conformational effect of these extensive deletions.

Mutant forms of EBNA2 accumulate to at least the same level as WT EBNA2 in transient expression in Burkitt's lymphoma cells. Since altered accumulation of mutant proteins could account for the poor transforming efficiency associated with *d*59-93 and *d*2-95, EBNA2 levels were evaluated by immunoblotting at 48 h after transfection of non-EBV-infected B-lymphoma cells with an expression vector having a WT or mutated EBNA2 ORF. All of the mutants were at least as stable as WT EBNA2, while *d*2-88, *d*2-95, *d*2-289, and *d*111-289 were more stable than WT EBNA2 (Fig. 3A, 4A, and data not shown). Some variability was observed among experiments in which *d*59-93 accumulated to slightly lower (Fig. 3A) or slightly higher (Fig. 4A) levels than WT. Thus, the poor marker-rescuing ability of *d*59-93 or *d*2-95 did not correlate with altered accumulation after transient expression.

The polyproline domain is not necessary for transactivation of the LMP1 promoter. Most mutations that affect the ability of EBNA2 to marker rescue transforming activity from P3HR-1 cells have a corresponding effect on the ability of the mutated protein to transactivate the EBV LMP1 promoter in transient transfection assays (3). In agreement with this expectation,

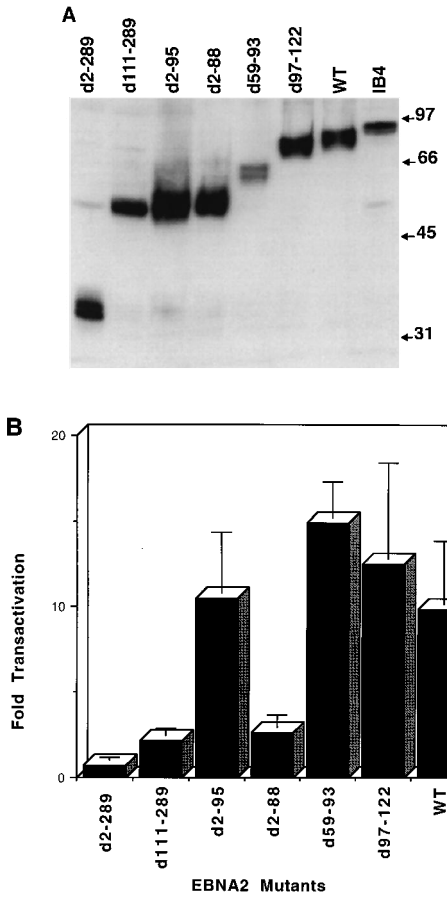

FIG. 3. Mutant or WT EBNA2 transient expression in B-lymphoma cell line BJAB (A) and effects on CAT expression from an LMP1 promoter (B). (A) BJAB cells transfected with an EBNA2 expression vector. After 48 h, lysates were obtained and assayed by Western blotting (immunoblotting) with EBNA2-specific monoclonal antibody PE2 and chemiluminescence. IB4 is an EBV-transformed LCL. The positions of molecular weight markers are shown. (B) A 5-μg amount of LMP1 promoter-CAT plasmid and 40 μg of EBNA2 expression plasmid were transfected into BJAB cells, and CAT activities after 48 h were assayed. Fold activity is relative to that of 40 μg of control expression plasmid without EBNA2.

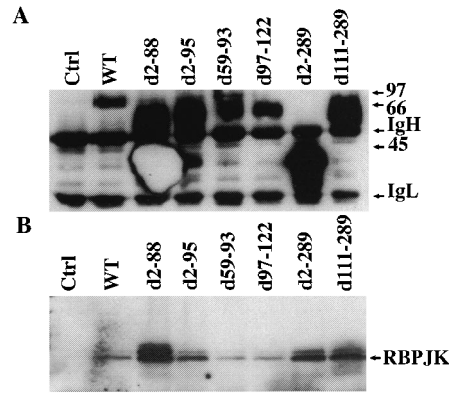

FIG. 4. EBNA2 N-terminal mutants interact with RBPJK. Expression vectors for mutant or WT EBNA2 were transfected into BJAB cells. After 48 h, EBNA2 complexes were immunoprecipitated from cell lysates by using the PE2 monoclonal antibody. The immunoprecipitates were electrophoresed through polyacrylamide gels and probed for EBNA2 (A) or RBPJK (B). Ctrl, immunoprecipitation from B cells that do not express EBNA2. The two bands common to all lanes in panel A (labeled IgL and IgH) are immunoglobulin light and heavy chains. The molecular mass of RBPJK is 63 kDa.

*d*2-289 and *d*111-289, which have a null transforming phenotype, were substantially deficient in LMP1 transactivation and *d*97-122, which is near WT in its transformation ability, was the same as WT in transactivation ability (Fig. 3B). However, *d*2-88 was near WT in transformation ability and was a poor transactivator of the LMP1 promoter, while *d*2-95 and *d*59-93 had null transforming phenotypes and were similar to or better than WT EBNA2 in LMP1 promoter transactivation (Fig. 3B). The data shown in Fig. 3B are representative from at least six independent transfections. The results of a low level of transactivation with *d*2-88 relative to WT EBNA2 are not due to toxicity secondary to the abundant accumulation of *d*2-88 in transiently transfected cells (see above), since a simian virus 40 promoter-driven β-galactosidase expression vector was used as the internal control in all transfection experiments, and β-galactosidase activities did not vary significantly. All CAT assays were normalized for the small variations in β-galactosidase expression that were observed. Thus, seven prolines confer decreased transactivation efficiency and increased transformation efficiency, on the basis of a comparison of *d*2-88 to *d*2-95.

LMP1 is expressed at WT levels in LCLs infected with *d*2-88 and *d*97-122 recombinants. To assess whether the ability of *d*2-88 recombinants to efficiently transform primary B lymphocytes was associated with higher levels of *d*2-88 expression or lower levels of LMP1 expression, as might be expected from the transient transfection assays, *d*2-88 recombinant-transformed B lymphocytes were compared with WT- and *d*97-122-transformed B lymphocytes with regard to EBNA2 and LMP1 expression. In both *d*2-88- and *d*97-122-infected LCLs, EBNA2 was usually expressed at levels somewhat higher than those in WT LCLs (Fig. 5A and data not shown). LMP1 was expressed at WT levels (Fig. 5B). Thus, despite the low level of transactivation activity of *d*2-88 in transiently transfected BJAB cells, LMP1 expression in LCLs infected with *d*2-88 EBNA2 recombinants was similar to that of *d*97-122 or WT EBNA2 recombinant-infected LCLs. The normal level of LMP1 expression in *d*2-88-infected cells could be due in part to increased EBNA2 accumulation, although higher accumulation was observed in

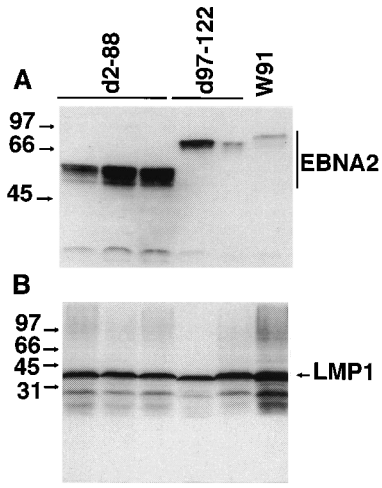

FIG. 5. EBNA2 and LMP1 expression in *d*2-88 and *d*97-122 recombinant virus-infected LCLs. Western blots of cell lysates were incubated with antibody specific for EBNA2 (A) or LMP1 (B), and antibody was detected by chemiluminescence. The positions of molecular weight markers are indicated.

transient transfections and LMP1 transactivation was abnormally low.

The failure of the polyproline deletion mutants to transform B cells is not due to a lack of interaction with RBPJK. To investigate whether the effect on transformation of the additional deletion of PPPPPPP that is characteristic of *d*2-95 as opposed to *d*2-88 could be due to an effect on RBPJK interaction, BJAB B-lymphoma cells were transiently transfected with WT or mutant EBNA2 expression plasmids, and the extent of association of EBNA2 with RBPJK was assayed by immunoblotting for RBPJK following EBNA2 immunoprecipitation (Fig. 4 and data not shown). All mutants associated with RBPJK, and the amount of RBPJK brought down in the immunoprecipitates roughly correlated with the amount of accumulated EBNA2. Most importantly, EBNA2 *d*2-88 and *d*2-95 were similarly abundant, and both associated with RBPJK at least as well as WT EBNA2. These results indicate that the polyproline domain is not critical for the interaction of EBNA2 with RBPJK.

DISCUSSION

The experiments described here, together with those reported previously (3), indicate that 222 of the 230 codons of the N-terminal half of EBNA2 are not essential for EBNA2 participation in EBV-mediated primary B-lymphocyte growth transformation (summarized in Fig. 1). Deletions of codons 2 to 88 or 97 to 122 had only minor effects on primary B-lymphocyte transformation efficiency, while deletions of codons 112 to 141 or codons 143 to 230 had been previously shown to have only moderate or minimal effects on transformation (3). The residual nondeleted codons 89 to 96 encode seven prolines and a glutamine. This glutamine is probably unimportant, since it is replaced by glutamic acid in EBV type 2 strains and in herpesvirus papio (13). In contrast, the prolines appear to be critical, since they cannot be specifically deleted (in *d*59-93) without a marked or complete loss of transformation efficiency. Thus, the seven prolines could constitute a domain critical for EBNA2 structure or for intermolecular interaction.

Several points can be made in support of the possibility that the seven prolines may effect an intermolecular association that is independent of the known role of EBNA2 as a transactivator of cell and viral genes with RBPJK and PU.1 regulatory sites. First, in EBNA2, the polyproline domain is nearly 200 amino acids away from the key domain for RBPJK interaction (8, 10, 24). Second, deletion of the polyproline domain did not have a significant effect on EBNA2 stability or an interaction with RBPJK in transient assays, compared with other mutants that had nearly WT levels of transforming activity. Third, deletion of the prolines did not destroy the ability of EBNA2 to transactivate the LMP1 promoter in transient transfection assays. Since PxxP domains are important in SH3 domain-mediated protein-protein interaction (25) and PPPPY has been implicated in binding to WW domains (2), a role for three to seven prolines in intermolecular interaction has some precedent.

However, the alternative hypothesis that the prolines do not mediate a unique intermolecular interaction but stabilize a broader EBNA2 interaction that is predominantly mediated by other essential domains is considerably more likely. First, seven prolines have rudimentary complexity, lack potentially interactive side chains, and are an unlikely mediator of protein-protein interaction. Nonproline residues are also critical for the SH3 and WW domain interactions, and other than a glutamine or glutamic acid residue, all other residues have been deleted from this site in EBNA2 without a substantial effect on transformation. Second, other mutations in EBNA2 have had significant effects on transformation without affecting transactivation in transient assays, only to be shown to be nonessential when deleted as smaller sequences. For example, deletion of codons 333 to 425 ablated transformation without affecting transactivation in transient assays. However, all subdomains of *d*333-425 could be deleted without more than a moderate effect on transformation (21). Third, while all EBNA2 response elements characterized so far have RBPJK sites and deletion of the polyproline domain appears not to affect RBPJK interaction, RBPJK is only one of two or more proteins that mediate EBNA2 interaction with response elements. The EBNA2 domain that interacts with RBPJK appears to be a simple oligopeptide sequence (10, 24), while the domain that interacts with PU.1 is more complex, and the interaction appears to be less stable (12, 25a). The polyproline domain could have a direct or indirect role in these EBNA2 effects.

The most intriguing aspect of these data is that they indicate that much of EBNA2 is dispensable for functionality in primary B-lymphocyte transformation. This includes the first 58 amino acids that precede the polyproline domain, most of the polyproline domain, and the domain that is immediately C terminal to the polyproline domain. This is surprising, since the first 58 amino acids and the 30 or so residues C terminal to the prolines include motifs that are highly conserved among the EBV types and primate lymphocryptoviruses (6, 13). This suggests that these sequences are important for modulating EBNA2 function in more complex natural environments in vivo.

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